PHOSPHORYLATION AND DEPHOSPHORYLATION OF RENAL BRUSH BORDER MEMBRANES BY PROTEIN KINASE AND PHOSPHOPROTEIN PHOSPHATASE*

Hussein ABOU-ISSA and Joseph MENDICINO

Department of Biochemistry, University of Georgia, Athens, Georgia 30602, USA

and

Fredrick LEIBACH and Dennis PILLION

Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30902, USA

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1. Introduction

Numerous investigations have shown that both gluconeogenesis and the uptake of amino acids in kidney are subject to hormonal regulation. Parathyroid hormone and cyclic 3′, 5′ AMP stimulate gluconeogenesis in isolated segments of renal tubules [1]. This hormone and cyclic 3′, 5′ AMP also stimulate the active uptake of amino acids in kidney cortex slices [2]. The active transport of amino acids in renal cortex has been examined [3]; however, the exact mechanism by which this hormone acts to stimulate the transport of amino acids has not yet been elucidated. It has been suggested that phosphoenzyme intermediates of the Na-K ATPase system may activate carrier proteins involved in the active transport of amino acids and sugars [4].

Bound protein kinases present in isolated swine kidney plasma membranes, nuclei and microsomes are able to phosphorylate endogenous proteins as well as glycogen synthetase and histone [5]. The present study demonstrates that a specific small acidic protein component of highly purified brush border membranes serves as a substrate for both endogenous bound protein kinase and added soluble protein kinase. Moreover, a phosphoprotein phosphatase isolated from swine kidney extracts catalyzes the dephosphorylation of this protein as well as the phospho forms of swine kidney glycogen synthetase and histone. Since these small acidic proteins have also been shown to be involved in binding of amino acids in these preparations [6,7] the present results suggest that the phosphorylation and dephosphorylation of this protein by enzymes present in the proximal tubule brush border membrane may play a role in the regulation of amino acid transport by epinephrine and parathyroid hormone in the tissue.

2. Materials and methods

Protein kinase was isolated from kidney homogenates as described previously [5]. The final preparation used in these studies was purified more than 3000-fold by affinity chromatography. The activity of the enzyme was assayed as described earlier [5]. The incorporation of ^{32}P into protein in brush border membrane preparations and the phosphorylation of exogenous protein substrates by protein kinase in these preparations was linear with time and increasing concentrations of enzyme under these conditions.

Phosphoprotein phosphatase purified more than 5000-fold from the same crude swine kidney homogenates was assayed by measuring the rate of release of trichloroacetic acid-soluble ^{32}Pi from histone or brush border membranes previously labelled with ^{32}P by incubation with protein kinase and [γ-^{32}P] ATP [8].

Brush border membranes were prepared from Sprague-Dawley rats by a modification of the procedure of Binkley et al. [6,7]. Kidneys, 10 g, were homogenized in 50 ml of 0.25 M sucrose, 0.01 M Tris–HCl, pH 8.0, 0.1 mM EDTA. The suspension was centrifuged at 350 *g* for 10 min and the pellet was resuspended in the same volume of buffer and rehomogenized. This preparation was centrifuged at 350 *g* for 15 min. The supernatants were combined and centrifuged at 8000 *g* for 20 min. The pellet was suspended in 6 ml of buffer and it was layered on a continuous 30 to 42% (w/w) sucrose gradient and centrifuged for 2 hr at 100 000 *g* in a SW 25 rotor. The brush border membrane fraction which collected at 36 to 38% sucrose was removed and the solution was diluted with 15 vol of 0.01 Tris–HCl, pH 8.0. The membranes were then collected by centrifugation at 27 000 *g* for 20 min. The pellet was resuspended in 3 ml of 0.25 M sucrose–0.01 M Tris–HCl, pH 8.0, 0.1 mM EDTA and the solution was layed on the 30 to 42% (w/w) sucrose gradient and the procedure described above was repeated. The final pellet was taken up in 1 ml of 0.01 M Tris–HCl, pH 8.0. Standard procedures for the assay of alkaline phosphatase, glucose 6-phosphatase, γ-glutamyl transferase, and ATPase in these preparations were used [6,9].

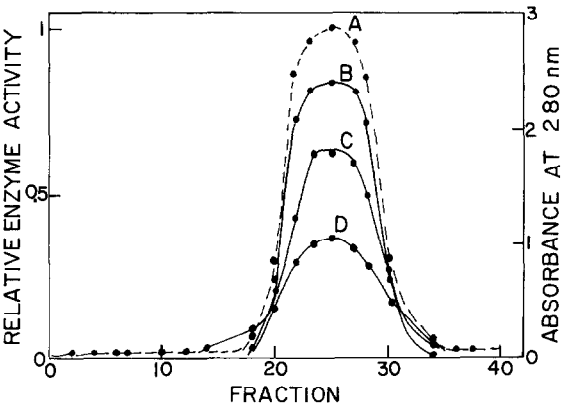

Fig.1. Sucrose density centrifugation patterns of enzyme activities associated with kidney brush border membranes. The standard method described in the text was used to sediment the membranes. Fractions 1 ml were collected and assayed. Curve A, protein by absorbance at 280 nm, Curve B, alkaline phosphatase, Curve C, protein kinase and Curve D, 5′-nucleotidase. The relative activity is expressed as a fraction of the fraction with the highest activity, in each case. The specific activities of alkaline phosphase, protein kinase and 5′-nucleotidase were about 0.9, 0.65 and 0.32 μmol/mg/min in all of the active fractions assayed.

3. Results and discussion

Kidney brush border membranes prepared by a number of different procedures all contain alkaline phosphatase, 5′ nucleotidase, γ-glutamyl transferase, ATPase and several other enzyme activities [6,9,10]. The relationship of the protein kinase activity associated with the brush border preparation used in the present studies to other enzyme activities known to be present in these membranes is shown in fig.1. The profile distribution of alkaline phosphatase and ATPase activity was coincident with the peak of protein kinase activity and brush border membranes following centrifugation in a 30 to 42% (w/w) sucrose gradient. The protein kinase was located at 36 to 38% sucrose, in the same position as the membranes and showed a constant specific activity relative to the amount of membrane protein present. The preparation was devoid of cytochrome *c* oxidase activity and RNA which indicated that it was essentially free of mitochondrial fragments and ribosomes. However, a large amount of glucose 6-phosphatase activity was found, which suggests that endoplasmic reticular membrane may be present in the preparation.

3.1. *Phosphorylation of kidney brush border membranes by protein kinase*

Endogenous protein kinase activity of isolated brush border membranes was measured using endogenous and exogenous proteins as the substrate for phosphorylation. Transfer of ^{32}P from [γ-^{32}P] ATP to an endogenous protein acceptor was observed, curve E, Fig.2, and the addition of exogenous purified soluble protein kinase increased the amount of ^{32}P transferred, curve D. Little or no incorporation was observed in the absence of magnesium ion, curve F. The rate of phosphorylation under these conditions was dependent on the amount of brush border membrane present, curve C compared to curve D. As seen in fig.2, the rate of phosphorylation of histone by endogenous protein kinase present in brush border membranes was at least 5 times greater than the rate

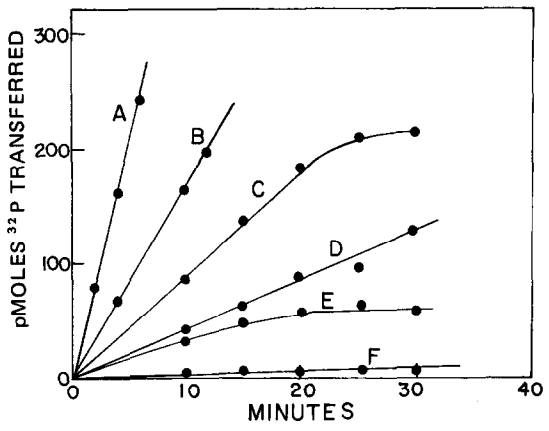

Fig.2. Phosphorylation of kidney brush border membranes by endogenous and purified protein kinase. The reaction mixture was incubated at 30°C and contained in 0.35 ml; 15 mM Tris–HCl, pH 7.0, 32 mM cysteine, pH 7.0, 2 mM $MgCl_2$, 2 mM [γ-^{32}P] ATP (5 × 10^7 cpm per μmol), 0.25 M sucrose. Curve A was obtained with 300 μg of membrane and 500 μg of calf thymus histone, Curve B with 150 μg of membrane and 500 μg of histone, Curve C with 300 μg of membrane and 10 μg of purified soluble protein kinase, Curve D with 300 μg of membrane and 5 μg of protein kinase, Curve E with 300 μg of membrane and Curve F with 300 μg of membrane and 5 μg of protein kinase with $MgCl_2$ omitted.

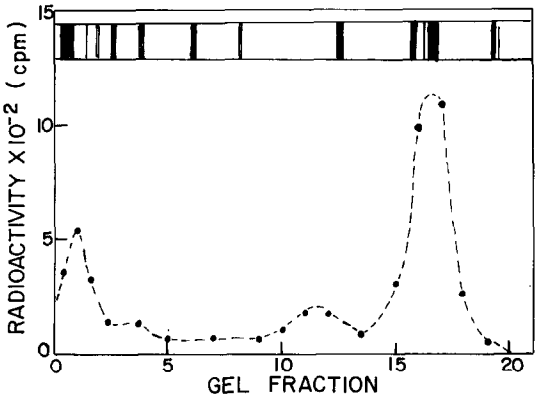

Fig.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified brush border membrane proteins. The ^{32}P-labelled membranes were prepared as described in the text using reaction mixture described in fig.1 except that 2 mg of membranes were incubated for 4 hr. Excess [γ-^{32}P] ATP was removed by dialysis against a solution containing activated charcoal. Membrane proteins were solubilized and electrophoresis was carried out as described in the text. Several gels run simultaneously were stained for protein with Coomassie Blue and others were frozen and sliced into sections for radio-activity determinations.

of phosphorylation of endogenous protein. These results demonstrate that the protein kinase associated with brush border membranes can phosphorylate both exogenous membrane proteins as well as added protein substrates. In other experiments ^{32}P-labelled brush border membranes were isolated and they were sedimented in a 30 to 42% sucrose gradient as described previously. The peak of radioactivity was found in the same area as the brush border membranes. The bound ^{32}P was not removed from the membranes by treatment with 0.5% Triton X-100, 0.9 M KCl, 6M guanidine–HCl or warm 10% TCA. However, the bound ^{32}P was almost completely hydrolyzed by 1 N NaOH and limited acid hydrolysis released ^{32}P-labelled phosphoserine. Thus, it may be concluded that the incorporated ^{32}P is present in covalent linkage with the serine residue of some membrane protein.

To further characterize the phosphorylated product ^{32}P-labelled membrane proteins were solubilized [11], and the samples were examined by polyacrylamide gel electrophoresis [12]. As seen in fig.3, most of the ^{32}P was found in a very acidic protein which migrated toward the end of the gel. Essentially the same pattern was obtained with endogenous membrane protein kinase or with the addition of exogenous purified soluble protein kinase. The minimum mol. wt of the ^{32}P-labelled protein estimated by electrophoresis of standard proteins dissociated under the same conditions was about 35 000. Based on this value and the specific activity of the [γ-^{32}P] ATP used in the phosphorylation reaction mixture about 0.3 mol of phosphate per mol of protein was incorporated. If the isolated membranes were first treated with purified kidney phosphoprotein phosphatase as described below and then phosphorylated with protein kinase about 0.7 mol of phosphate per mol of protein was incorporated. These results suggest that a specific protein present in brush border membranes is phosphorylated by both endogenous protein kinase present in the membrane and by added soluble protein kinase.

3.2. *Dephosphorylation of kidney brush border membranes*

The release of ^{32}Pi from labelled brush border membranes by endogenous and added purified kidney phosphoprotein is shown in fig.4. Only a small amount of ^{32}P was hydrolyzed by endogenous phosphatase, curve

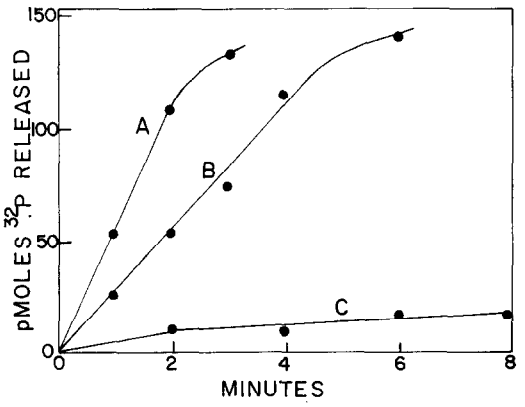

Fig.4. Release of ^{32}Pi from labelled brush border membranes by phosphoprotein phosphatase. The reaction mixture was incubated at 30°C and contained in 0.8 ml; 0.05 M imidazole–HCl, pH 7, 30 mM 2-mercaptoethanol, 0.1 mg of bovine serum albumin, 2 mg of glycogen, 1 mM $MnCl_2$. The release of acid soluble ^{32}Pi was determined as described in the text. Curve A was obtained with 300 μg of ^{32}P-labelled membrane containing 150 pmol of ^{32}P and 20 μg of swine kidney phosphoprotein phosphatase, Curve B with 300 μg of ^{32}P-labelled membranes and 10 μg of phosphoprotein phosphatase, Curve C with 300 μg of ^{32}P-labelled membranes.

C. However, the addition of purified phosphoprotein phosphatase caused a rapid hydrolysis of the membrane-bound ^{32}P and as seen in the figure the rate was dependent on the concentration of phosphoprotein phosphatase added. After about 10 min under these conditions essentially all of the ^{32}P present in this preparation, 150 pmol, was released as ^{32}Pi by the action of the added phosphoprotein phosphatase.

The experiments described demonstrate that kidney protein kinase and phosphoprotein phosphatase which were previously shown to act on glycogen synthetase, histone and other soluble protein substrates [5] also act on a specific small acidic protein bound to the brush border membranes in this tissue. Small acidic proteins have also been implicated in the binding of amino acids and possibly amino acid transport in these membrane preparations [12]. Therefore, it is possible that a phosphorylation–dephosphorylation mechanism may be involved in the activation of amino acid transport as well as glyconeogenesis in this tissue. The correlation of amino acid uptake and phosphorylation of proteins in kidney brush border and plasma membrane preparations is currently being examined.

Acknowledgements

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